Use of the Enzyme-Linked Immunosorbent Assay (ELISA) in Immunotoxicity Testing

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Based on an earlier described macromethod for the routine measurement of IgM and IgG in rat sera, a mechanized micro enzyme-linked immunosorbent assay (ELISA) was developed. The assay was performed in the wells of microtiter plates thus minimizing the quantities of reagents and antisera needed. Data on reproducibility of the assay and calculation of IgM and IgG levels are provided.

For the functional assessment of the humoral immunity of the rat, ELISA is a powerful tool. In an earlier report, assays for the titration of thymus-independent IgM antibodies to *E. coli* LPS and the IgM and IgG response to the thymus-dependent antigen tetanus toxoid were described. More recently it was shown that the antigen ovalbumin elicits a thymus-dependent IgM, IgG and IgE response which could be readily measured with the enzyme immunoassay, as well as a delayed-type hypersensitivity reaction. As the optimum ovalbumin concentration for both types of reactions was the same, it is concluded that the ovalbumin model offers the advantage that both humoral and cellular immunity can be studied simultaneously in the same animal.

Introduction

In clinical immunology, serum immunoglobulin (Ig) levels are routinely determined parameters. Also in the field of immunotoxicology, determination of main Ig classes may provide valuable information about chemically-induced alterations of the humoral immunity (1). A routine method for measurement of serum Ig levels is by single radial immunodiffusion (SRID). An alternative method is the enzyme-linked immunosorbent assay (ELISA). After the introduction of the ELISA for the determination of rabbit IgG (2), this technique has now become used in a large and still increasing number of applications which include measurements of antigens, haptens and antibodies (3,4).

In an earlier report, we have described a "sandwich" ELISA for the routine measurement of total

IgG and IgM levels in rat sera (5) using a mechanized macro-ELISA system (6). In the "sandwich" ELISA, antigen (e.g., rat IgM) reacts with excess solid-phase antibody. After washing, the bound antigen is reacted with excess enzyme-labeled antibody. After further washing, the bound label is assayed, providing a direct measure of the amount of antigen present. IgM and IgG measurements by ELISA correlate well with results obtained with SRID in sera of rats of various age groups. In addition, the precision of the enzyme immunoassay was the same as that obtained with the SRID. However in comparison with the latter method, the ELISA was less time-consuming and the amount of antiserum needed was one order of magnitude lower, and two orders of magnitude lower in the mechanized micro-ELISA that will be described here.

For the functional analysis of the humoral immunity, ELISA is also a powerful tool. In the ELISA for antibodies, antigen is coupled to a solid phase, incubated with the test serum, and the amount of antibody bound to the solid phase is quantified by

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peroxidase-labeled class-specific anti-immunoglobu-lins. In an earlier report (5), ELISA and the passive hemagglutination test were compared in the titration of rat antibodies against *E. coli* lipopolysaccharide (LPS) and tetanus toxoid. Generally ELISA was more sensitive than the hemagglutination reaction and has the major advantage that, by using class-specific enzyme-labeled antisera, both IgM and IgG antibodies could be measured. Based on the similarity of the primary and secondary immune response to LPS, suggesting that immunologic memory did not develop after primary immunization (5), and the similarity of the IgM response to *E. coli* LPS in athymic nude rats versus thymus-bearing normal littermates (7), it is evident that the humoral response of the rat to *E. coli* LPS is a thymus-independent phenomenon. This means that so-called helper T-cells are not involved in the generation of an immune response. Based on the rapid acceleration of the synthesis of IgG antibodies upon secondary immunization with tetanus toxoid (5) and the absence of IgM and IgG antibody responses in athymic nude rats (7), tetanus toxoid represents a thymus-dependent antigen.

In the present report, a mechanized micro-ELISA is described for the routine measurement in toxicity studies of rat IgM and IgG, and further improvements and data on reproducibility and calculation of IgM and IgG levels are given. In addition, results of a recent study (8) are discussed showing that the antigen ovalbumin elicits a thymus-dependent IgM, IgG and IgE response as well as a delayed-type hypersensitivity response. As the optimal antigen dose for the humoral and cell-mediated reactions is the same, this model offers the advantage that both types of function tests can be studied simultaneously in the same animal.

Antisera and Conjugates

Goat-anti-rat IgM (GaRaIgM) was obtained commercially from Nordic Pharmaceuticals and Diagnostics (Tilburg, The Netherlands). Anti-rat IgG was prepared by hyperimmunization of a sheep with purified IgG emulsified in complete Freund's adjuvant (Difco). The antiserum to IgG (SaRaIgG) was rendered monospecific by absorption with F(ab')$_2$ fragments, obtained by peptic digestion of rat IgG. Anti-rat IgE was prepared by hyperimmunization of a sheep with IgE emulsified in complete Freund's adjuvant. Rat IgE was isolated by salt fractionation, gel filtration and ion-exchange chromatography from serum of Lou rats bearing plasmacytoma IR 162. The antiserum (SaRaIgE) was absorbed with normal rat and rat IgA myeloma (IR 22) serum and fractionated over Sephadex G-200 (Phar-

macia, Uppsala, Sweden) to isolate the 7S fraction devoid of immune complexes and most of the excess of rat serum proteins. Immunoglobulin fractions of the three antisera were prepared by caprylic acid factionation (9).

The antisera against IgM, IgG and IgE appeared to be monospecific as tested by gel precipitation (Ouchterlony technique). In addition, GaRaIgM and SaRaIgG were tested for specificity in the more sensitive ELISA. For that purpose, wells of polystyrene microplates were coated with GaRaIgM or SaRaIgG, incubated with serially diluted normal rat serum, and finally incubated with peroxidase-labelled SaRaIgG and GaRaIgM. In this test system, the GaRaIgM appeared to contain less than 1% cross-reacting (anti-IgG) antibodies, whereas anti-IgM antibodies were virtually absent (< 0.01%) in the SaRaIgG. A similar specificity (less than 0.01% cross-reacting anti-IgG antibodies) could be demonstrated for SaRaIgE when the heat lability of rat IgE antibodies was utilized. When peroxidase-labelled SaRaIgE was used, no antibodies were detected in high-titered anti-OA sera (^{2}log IgG titers of approximately 15) when the rat sera were heated for 2 hr at 56°C.

Horseradish peroxidase (PO), type VI, R.Z.3, was obtained commercially from Sigma Chemical Co. (St. Louis, USA). PO was conjugated to the antisera by the method of Nakane and Kawaoi (10).

Equipment for Micro-ELISA

In the mechanized system (Fig. 1), conventional polystyrene microtiter plates with 12 × 8 flat-bottomed wells were used for through the plate reading of extinction values. A detailed description of the dispenser and simple plate washer is given by Ruitenberg and Brosi (11). Extinctions were measured on a commercially available multiple-channel photometer for vertical measurement (Titertek Multiskan, made for Flow Laboratories by Eflab Oy, Helsinki, Finland). Details of the performance of this photometer have been published by Ruitenberg et al. (12).

Ruitenberg and Brosi (11) also provide commercial sources for equipment for manual dispensing, for an automated washer, and for manual and automated processing (multiple dispenser, washer and photometer).

"Sandwich" Micro-ELISA for Quantification of Rat IgM and IgG

The procedure for the micro-ELISA was largely the same as for the macroassay in which polysty-
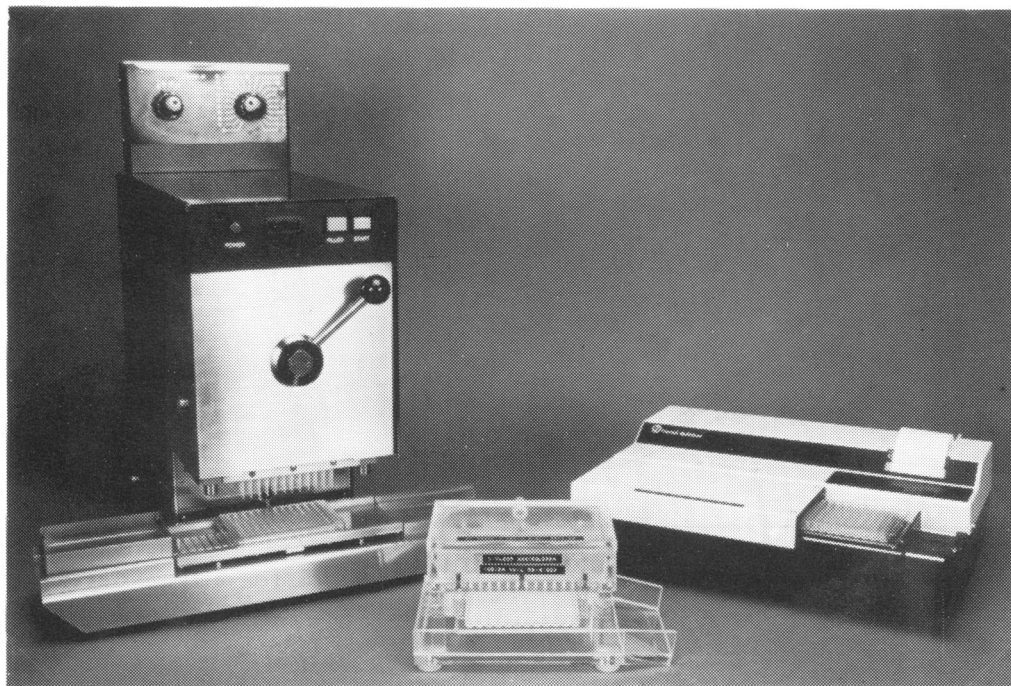
FIGURE 1. Dispenser, plate washer and multichannel photometer for vertical through-the-plate reading of microplates.

rene tubes were used as antigen carriers (5). Changes that did improve the test will be discussed below.

Wells of polystyrene microtiter plates were coated by adding 100 μl of anti-immunoglobulin solution (15 μg protein/ml) in sodium carbonate buffer (0.1 M; pH 9.6) with 0.02% NaN_3 as preservative. After incubation at 37°C for 18 hr, in which time the added fluid evaporated, plates were washed by using tap water of neutral pH containing 0.05% Tween 20. For the IgM and IgG analysis sera (reference serum of pooled adult rat serum or pooled serum of control animals as well as the test sera) were diluted with phosphate buffered saline (PBS; 0.01M; pH 7.2) containing 2% bovine serum albumin (BSA) to which 0.05% Tween 20 was added. Quantities of 100 μl were added to each well, and plates were incubated under rotation at 37°C for 1 hr. After washing, 100 μl of the conjugate (in an optimal dilution of PBS with 2% BSA and 0.05% Tween 20) was added. Plates were incubated under rotation at 37° C for 2 hr. Excess conjugate was removed by washing. Finally, the amount of enzyme bound to the wells was determined by adding 100 μl of the conventional substrate for ELISA (5-aminosalicylic acid and H_2O_2). Earlier (5), the substrate was prepared by dissolution of 80 mg of 5-aminosalicylic acid in 100 ml of distilled water at approximately 70°C; directly prior to use the pH of this solution was brought to 6.0 with 1N NaOH. As

shown in Figure 2, dissolution of the substrate in 0.05M maleate buffer of pH 6.0 markedly improved the slope of the standard curve. Also the concentration of H_2O_2 strongly influenced the assay. Earlier, to 9 ml of the 5-aminosalicylic acid solution 1 ml of 0.05% H_2O_2 was added. As shown in Figure 3, the
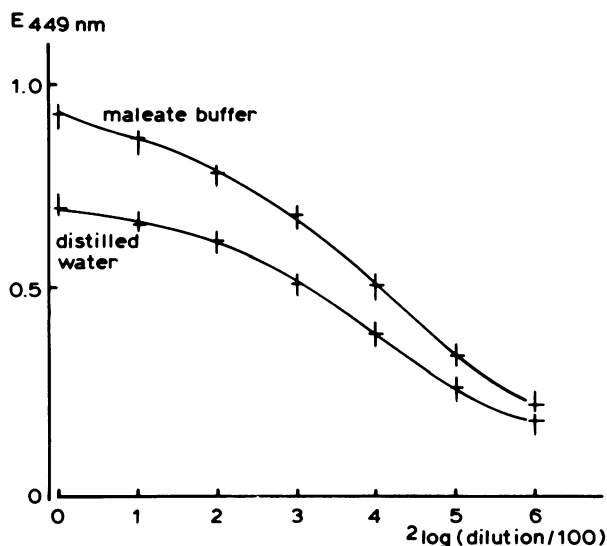

FIGURE 2. Difference in IgM standard curve by dissolution of the substrate 5-aminosalicylic acid in distilled water or in 0.05M maleate buffer (pH 6.0).

E 449 nm

1.0

0.05
0.10

0.20

0.025

15 min.

0.5

0

0   1   2   3   4   5   6
^{2}log (dilution/100)

E 449 nm

0.10

0.05

0.20

1.0

0.025

30 min.

0.5

0

0   1   2   3   4   5   6
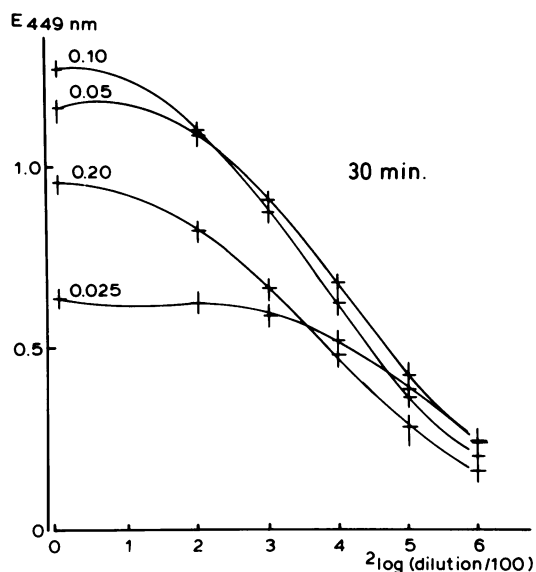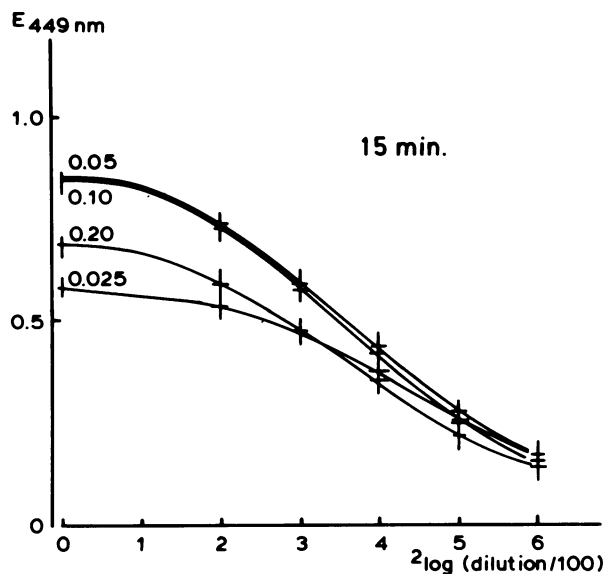^{2}log (dilution/100)

FIGURE 3. Effect of different hydrogen peroxide concentrations (0.025-0.2%) added to the substrate on extinction values in the IgM micro-ELISA: (top) after 15 min; (bottom) after 30 min incubation.
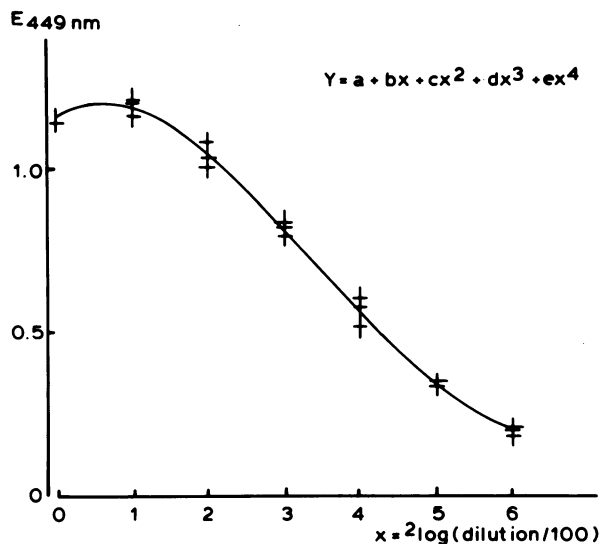

E 449 nm

$Y = a + bx + cx^2 + dx^3 + ex^4$

1.0

0.5

0

0   1   2   3   4   5   6
$x = {}^2$log (dilution/100)

FIGURE 4. Standard curve calculated by least-squares method for measuring rat IgM by micro-ELISA.

concentration of H_2O_2 is critical, as a twofold lower (0.025%) or fourfold higher (0.20%) concentration of H_2O_2 caused a pronounced decrease in extinction values at low serum dilutions, resulting in a standard curve with a flat slope. Since the concentration of H_2O_2 in the stock solution decreases in time, a concentration of 0.10% H_2O_2 was chosen to be added to 5-aminosalicylic acid, and 100 μl amounts of the freshly prepared substrate were added to the wells. After incubation at room temperature for an optimal period of 30 min (Fig. 3), extinctions were measured on the multichannel photometer.

For the quantification of IgM and IgG levels, standard curves consisting of seven twofold serial dilutions of a reference serum or a pooled serum of the control group, were calculated by least-squares fit of the fourth-degree polynomial (Fig. 4). Test sera were analyzed in one dilution. In addition, a conjugate control was included (i.e., anti-immunoglobulin-coated well, to which conjugate was added and after proper incubation and washing, the sub-

Table 1. Statistical analysis for estimating differences in serum IgM levels in control and hexachlorobenzene-treated rats.[a]

Group	IgM level in standard serum, %					Mean	SD	F ratio	N
1	28.2	30.95	48.8	35	24.15	31.4938	7.7271		8
	25.7	29.85	39.3						
2	31.25	30.95	27.45	26.45	34.15	34.1125	9.1273	1.3952	8
	52.85	27.5	42.3						
3	33.9	54	39.05	63.75	54.15	47.5313	13.5300[b]	3.0659[c]	8
	44.45	27.35	63.6						

[a]IgM HCB PO 78/70.
[b]Significance by Student t-test: $p < 0.05$.
[c]Significance by Welch t-test: $p < 0.05$.

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strate). In order to eliminate variations between plates, a standard curve was included in each plate. This means that per microtiter plate, 21 wells were used for the standard curve, 3 for the conjugate control, and 72 for the test samples (triplicate analyses of 24 rats). Differences in group mean values were estimated by Student's t-test, and results expressed either as percentage of the IgM or IgG level in the standard serum (Table 1) or as percentage of the control serum. The calculations were performed on a BASIC programmable topdesk computer.

The reproducibility of the enzyme immunoassay for IgM and IgG was determined by comparing extinction values of twofold dilutions of the reference serum, determined in five sessions spread over a 4-month period. As shown in Figure 5, extinction values determined on different days were nearly identical for both IgM and IgG, demonstrating the excellent reproducibility of the assays. The data shown in Figure 5 were obtained by macro-ELISA. Preliminary data of the micro-ELISA however are indicative of a high reproducibility as well.

A problem encountered in the use of micro-ELISA which may influence the results lies in the position of the wells in the microtiter plates. As shown in Figure 6, the wells at the edge of the microplates showed higher extinctions, and wells in the center showed lower extinction values. This difference may originate from inhomogenicity in the polymerization process of the polystyrene plate, which in turn may influence the coating efficiency. Effects of well position however are also known from lymphocyte transformation studies in microplates in which no coating is involved. The systematic influences of well position on the outcome of the test is reduced by an at random distribution of the triplicates of each sample. However, the variation due to well position is small when compared to the variation in serum IgM and IgG levels between individual animals. This is demonstrated by the finding that IgG levels of a group of random-bred rats of the Wistar strain, bred and housed under SPF conditions, showed a marked variation (Fig. 7). Also shown in this figure is the dramatic effect of a virus infection on the serum IgG level when rats bred under SPF conditions were housed under conventional conditions after weaning. For example, serum IgG levels of one group housed under conventional conditions and all serological positive for pneumonia virus of mice, were 150% increased in comparison with SPF littermates. Besides the induction of specific antibodies, which may markedly increase Ig levels, the microbiological status of experimental animals is extremely important in immunotoxicity testing as various infectious agents
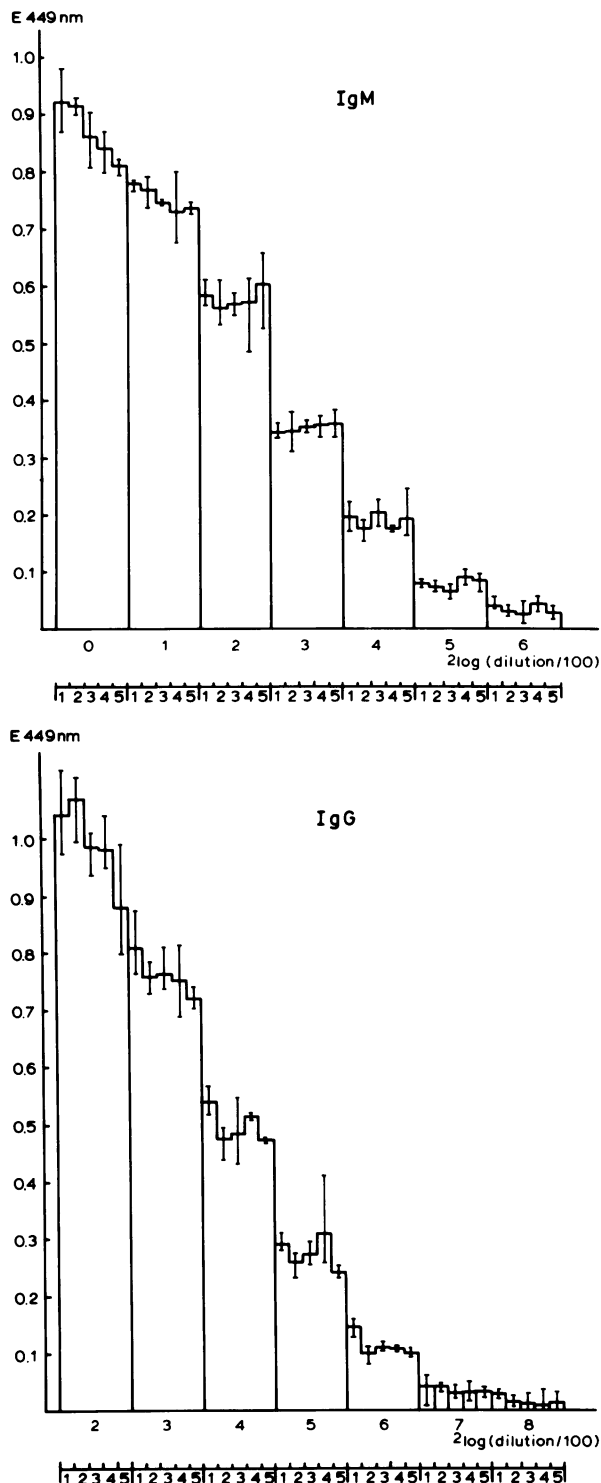

FIGURE 5. Reproducibility of extinctions of twofold serial dilutions of the standard serum as measured with the macro-ELISA. Analyses of (a) IgM and (b) IgG were performed in five sessions spread over a 4-month period. Results of triplicate analyses are expressed as mean and extreme values.
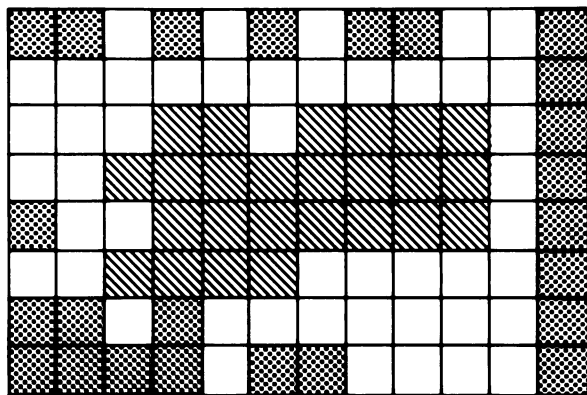
FIGURE 6. Effect of well position in microplate on extinction values of rat serum tested in one dilution. Results of IgM measurements are given as a percentage of the deviation of the mean.
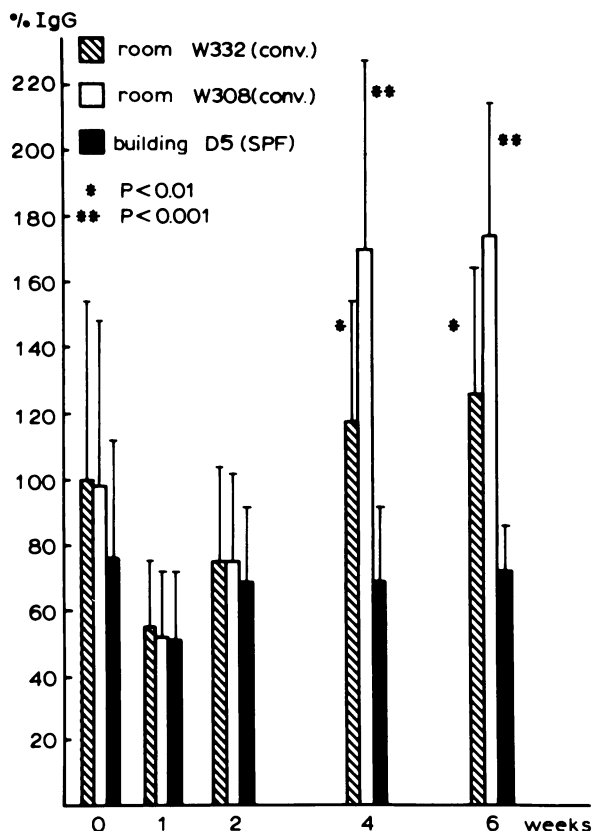
dev. ≥ +4%   -4% < dev. < +4%   dev. ≤ -4%

FIGURE 7. Serum IgG levels of rats bred under SPF conditions, and after weaning under conventional or SPF conditions (means ± SD of 9-10 rats per group). Note the large variation of IgG levels in SPF animals and the strong increase in IgG levels of rats housed under conventional conditions and infected with pneumonia virus of mice (13).

do modify the immunological responsiveness of the host (14). The outcome of an experiment may be influenced by such an infection, particularly when function tests of the immune system are carried out.

ELISA for Determining IgM, IgG and IgE Titers to Ovalbumin

The immunization procedure and the micro-ELISA are described in detail elsewhere (8). In short, rats are immunized by a subcutaneous injection in the hind footpads with an optimal dose of 100 μg ovalbumin (crystallized five times; Serva Feinbiochemica, Heidelberg, Germany) dissolved in 0.05 ml PBS and emulsified in 0.05 ml of complete H37Ra adjuvant (Difco Laboratories, Detroit, Michigan). For the determination of anti-ovalbumin antibodies routinely day 10 and 22 sera are used.

The enzyme immunoassay was performed with the dispenser and washer shown in Figure 1. Polystyrene microplates were coated with 5 μg ovalbumin/ml sodium carbonate buffer, $0.1M$, pH 9.6. After coating for 2 hr at 37°C, the microplates were washed with tap water of neutral pH containing 0.05% Tween 20. Serial twofold dilutions of the rat sera were prepared in PBS ($0.01M$, pH 7.2) containing 0.05% Tween 20. Since a cross-reaction was observed between antibodies against OA and bovine serum albumin, the latter protein was not added to the serum diluent or to the conjugate. Microplates containing 100 μl of the diluted sera were incubated at 37°C for 1 hr. After being washed, 100 μl of conjugate was added after proper dilution with PBS ($0.01M$, pH 7.2) containing 0.05% Tween 20. Microplates were incubated under rotation at 37°C for 2 hr. The trays were washed again, and 100 μl of substrate (5-aminosalicylic acid and H_2O_2) was added. After incubation at room temperature for approximately 1 hr, the reaction product was evaluated visually and expressed as ^{2}log of the highest dilution giving a positive color reaction.

As shown in Figure 8, the optimal antigen dose for functional assessment of the IgM, IgG and IgE antibody response lies around 100 μg ovalbumin. It also appears that the same concentration is optimal for the delayed-type hypersensitivity (DTH) reaction as parameter of the cell-mediated immunity. Therefore, these function tests can be combined in the rat, which decreases the number of experimental animals needed. The absolute thymus dependency of the humoral response to ovalbumin is clearly demonstrated by the negative ELISA IgM and IgG reactions in 1:2 diluted sera of athymic

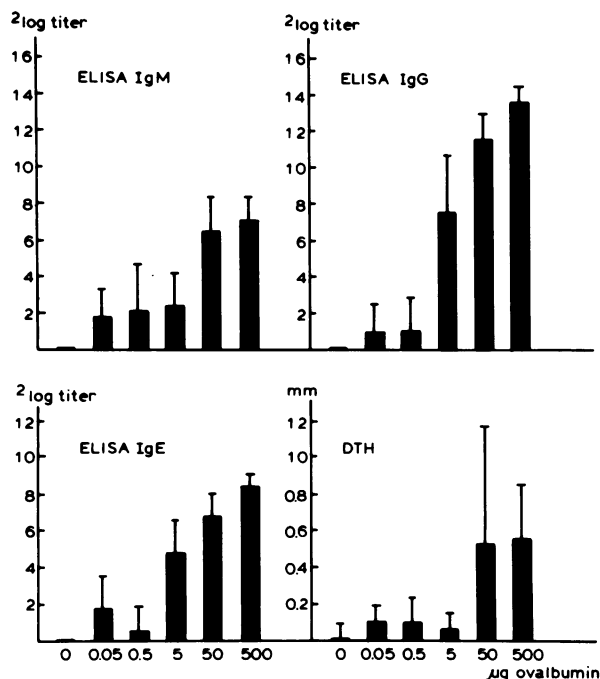**Environmental Health Perspectives**

FIGURE 8. ELISA IgM, IgG and IgE titers and delayed-type hypersensitivity (DTH) reactions as measured 3 weeks after SC immunization of Wistar rats with various concentrations of ovalbumin, emulsified in H37Ra adjuvant. DTH reactions are expressed as differences in thickness of 24 hr skin reaction between ovalbumin-challenged and control ear (8).

rats, whereas thymus-bearing littermates had a high response (serum dilutions of 1:30,000 still yielded a positive IgG reaction) (7).

Instead of endpoint titration, IgM, IgG and IgE antibodies to ovalbumin can also be quantified by measurement of extinction values in twofold serially diluted sera. As shown elsewhere (8), at certain dilutions of the antisera to ovalbumin, extinctions were measured that approached a linear function when plotted against the dilution, while within the IgM, IgG or IgE assays, the slopes of the linear area were the same. The same holds true for the measurement of IgM antibodies against *E. coli* LPS (5). By such an approach, the discriminatory power of the enzyme immunoassay increases which may be of importance when subtile effects on the humoral immunity are studied.

Like all immunological tests in which anti-immunoglobulins are used, the specificity of the enzyme immunoassay largely depends on the quality of the antisera and conjugates used. Conventional commercial antisera frequently do not meet the high standard needed. However, it can be expected that in the near future high quality monoclonal antisera, obtained by the hybridoma technique, will be com-

mercially available. Because of simplicity and sensitivity, coupled to a very economic use of antisera, it can be expected that also in the toxicology area enzyme immunoassays will become routine methods to quantify antigens and antibodies. There is the added advantage that the (enzyme-labeled) antisera can also be used in histology, e.g., for the immunocytochemical demonstration of plasma cells.

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